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A New Immunomodulatory Function of Low-Viscous Konjac Glucomannan with a Small Particle Size: Its Oral Intake Suppresses Spontaneously Occurring Dermatitis in NC/Nga Mice

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Key Words
Atopic dermatitis · Interferon-γ · IgE · Konjac glucomannan · NC/Nga mice · Particle size

Abstract
Background: Konjac glucomannan (GM) is a well-known dietary fiber with various beneficial functions: the higher viscosity displayed the stronger potency. However, the high-viscous GM powders, ordinary konjac powder and highly purified GM were mostly unsuitable for the application to various food industries. Our aims are to develop new physiological functions of low-viscous GM powder, pulverized GM or re-granulated fine GM, using a murine model of atopic dermatitis. ***Methods:*** Male 4-week-old NC/Nga mice were fed for 8 weeks on diets containing 5% of two high-viscous and two low-viscous GM powders, respectively. ***Results:*** Striking suppression against the aggravation of dermatitis, the increase in scratching behaviors, and the rise in IgE levels was recognized only in mice fed on the pulverized GM diet, but not in mice fed on the other GM diets or a control diet. Eczema prevention in the fine GM-fed mice was accompanied by a significant decrease in their plasma IFN-γ levels, a positive regulatory cytokine for atopic skin inflammation. ***Conclusion:*** Only the pulverized GM possessed the ability to suppress the development of dermatitis in NC/Nga mice. This is the new immunomodulatory function of low-viscous GM with a small particle size.

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Introduction

A Japanese traditional food, konjac, which is rich in glucomannan (GM), a kind of dietary fiber, has been manufactured with konjac powder (KP) obtained from the tubers of *Amorphophallus konjac*. Konjac GM is a highly viscous polysaccharide composed of glucose and mannose residues in the molar ratio of 2:3 with β1–4 linkages.

In the 1970s, epidemiological studies suggested that dietary fibers may play important roles in inhibitory

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effects on colon cancer [1, 2], cardiovascular disease [3], and diabetes mellitus [4]. Since then, numerous studies have gradually manifested that such water-soluble dietary fibers as konjac GM possess various beneficial functions: improvement in hyperglycemia, hyperlipidemia, and hypertension, i.e. three major risk factors of coronary heart disease in type 2 diabetes patients [5, 6], and the inhibition of the increments in serum glucose and insulin levels [7, 8]. In most cases, the higher viscosity exhibited the higher efficacy of the function. Therefore, high viscosity has been considered to be an essential factor in these physiological effects.

In part of the studies, the effects of dietary fibers on splenocytes or mesenteric lymph node (MLN) lymphocytes were analyzed. Oral administration of dietary fibers, konjac GM, pectin, and chitosan, decreased in serum IgE levels while serum IgA and IgG levels increased, and especially pectin-feeding enhanced IFN-γ production of MLN lymphocytes in Sprague-Dawley rats [9]. Water-soluble dietary fibers indirectly regulated immunoglobulin production of lymphocytes, and their activities were dependent on the molecular size [10].

Due to the properties similar to ordinal dietary fibers, konjac GM has partly been used as healthy food. However, the nutritional supplementation of high viscous GM is restricted on account of the low solubility that requires long time intervals to reach peak viscosity. To circumvent the restriction, we prepared highly specific surface area of the GM molecule that would be expected to possess some extent of these well-known physiological activities. However, apart from the physicochemical properties, physiological function of low-viscous fine GM thus prepared has been scarcely investigated yet. Accordingly, an inquiry into a new additional or sole function of the low-viscous GM powder would be indispensable to expand the market.

NC/Nga mice have recently been introduced as a murine model for human atopic dermatitis (AD). The mouse develops spontaneously AD-like skin lesions accompanied by typical itching behavior and hyper-IgE production under conventional conditions [11]. Using this AD model, we detected a new immunomodulatory function of the low-viscous pulverized GM that could suppress the development of AD-like symptoms. This ameliorative effect was never observed in ordinal high-viscous GM feeding. To determine whether the viscosity is the main factor responsible for the new function, fine GM (S-P) was re-granulated to an appropriate particle size (S-gw) maintaining low viscosity.

Evidence has accumulated that IFN-γ production from Th1 cells plays an important role in the pathophysiology of AD. Increased mRNA expression of IFN-γ was significantly suppressed after successful therapy of AD patients [12]. A similar local overexpression of IFN-γ and accumulation of IFN-γ-producing T cells has been demonstrated in NC/Nga mice [13]. Administration of transforming growth factor (TGF)-β_1 or royal jelly feeding suppressed AD-like dermatitis in NC/Nga mice via the downregulation of IFN-γ [14, 15].

This study demonstrates a novel physiological function of low-viscous GM to suppress the development of dermatitis through the systemic downregulation of IFN-γ, and describes the important role of its small particle size in AD suppression.

Materials and Methods

Preparation of Konjac GM Powders

Four kinds of konjac GM powders, konjac powder (KP), highly purified GM powder (PA, PROPOL®), pulverized GM powder (S-P), and re-granulated fine GM powder (S-gw), prepared by Shimizu Chemical (Hiroshima, Japan) were used in this study. KP was manufactured by reducing the tubers of *Amorphophallus konjac* to a powder. PA was prepared after polishing KP over and over in ethanol to eliminate impurity. After a few purifications of KP with ethanol, fine S-P was obtained by crushing. S-gw was provided by re-granulating S-P in a fluidized-bed granulator.

Physicochemical Properties of the Various Konjac GM Powders

The contents of dietary fiber in konjac GM powders were quantified according to the standard method recommended by the Pharmaceutical Society of Japan [16]. The changes in absolute viscosity of 1% konjac GM solutions with time were monitored at 25°C by a B-type viscometer (Tokimec, Tokyo, Japan). The mean particle size and size distribution of these GM powders were measured by a Laser Micron Sizer LMS-24 (Seishin Enterprise, Tokyo, Japan).

Animals and Diets

Male 4-week-old NC/Nga mice (conventional grade) purchased from Japan SLC (Shizuoka, Japan) were divided into five groups of 5 rodents each and maintained under conventional conditions for 8 weeks on a control diet (MF diet, Oriental Yeast, Tokyo, Japan) or a konjac GM diet [MF diet containing 5% (w/w) konjac GM powder] ad libitum. All animals were housed in a room kept at 22 ± 2°C with a 12-hour light/12-hour dark cycle.

Evaluation of Skin Involvement

Clinical features of the skin and the severity of dermatitis in NC/Nga mice were scored once a week by an observer. A score for AD-like symptoms was evaluated according to the amount of itching, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness on the face, ears, neck, and body. Each of the five symptoms was graded from 0 to 3 (none, 0; mild, 1; moderate, 2, and severe, 3). Clinical severity of dermatitis was expressed as the sum of the scores obtained for the five symptoms.

After scoring skin involvement, scratching behaviors of 5 NC/Nga mice in a cage were recorded for 30 min using an automatic video camera. The videotape was inspected to determine the scratching of the face, ears, neck, and body with the hind paws. NC/Nga mice showed various itching behaviors, short to long duration of the scratching, so that a series of such behaviors was counted as one time incident. Because the total number of scratching in 5 NC/Nga mice per 20 min was counted, data were not analyzed statistically.

Measurement of Plasma Immunoglobulins and IFN-γ

Blood samples were collected once every 2 weeks, and the total plasma IgE levels were measured by a sandwich ELISA using two kinds of anti-mouse IgE mAb (PharMingen, San Diego, Calif., USA). At 12 weeks of age, the plasma IgG1 and IgG2a levels were also measured by a sandwich ELISA using a mouse immunoglobulin isotyping kit mAb (PharMingen). The concentrations of IFN-γ in the plasma were also measured by a sandwich ELISA using a pair of mAbs of rat anti-mouse IFN-γ (PharMingen).

Measurement of Cytokine Production in Cell Culture

At 12 weeks of age, spleen cells were collected from NC/Nga mice that had been fed on either the control diet or the konjac GM diets, and the cells were treated with lysis buffer (150 m*M* NH_4Cl, 15 m*M* $NaHCO_3$, 0.1 m*M* EDTA2Na, pH 7.3) to lyse red blood cells. After washing 3 times with PBS, spleen cells (2×10^6 cells/ml) were stimulated with immobilized anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies (PharMingen) in RPMI-1640 medium (Sigma, St. Louis, Mo., USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μ*M* 2-mercaptoethanol, 10% fetal bovine serum (Gibco BRL, Life Technologies Oriental, Tokyo, Japan) at 37 °C for 72 h in 5% CO_2/95% air. IL-4 and IFN-γ levels in culture supernatant were detected by a sandwich ELISA using two pairs of mAbs of rat anti-mouse IL-4 and rat anti-mouse IFN-γ (PharMingen).

Statistics

Statistical analysis of the skin severity score between the groups was performed using the Mann-Whitney U test, and intergroup comparisons of the other parameters were performed using Student's t test. $p < 0.05$ was accepted as the level of significance.

Results

Physicochemical Properties of Low-Viscous Konjac GM Powders

The GM content, mean particle size, viscosity, and solubility of GM powder (S-P) obtained by pulverization of ethanol-purified KP were compared with those of ordinal KP, highly purified PA, and re-granulated S-gw (table 1). The particle size of pulverized S-P was distributed as a peak centered at 120 μm with a shoulder at 30–40 μm, although the particle size of the other GM powders showed relatively narrower distribution. In low-viscous GM powders, S-P and S-gw, the time to reach a constant viscosity was shorter.

Table 1. The physicochemical properties of konjac GM

Konjac GM	Physicochemical properties			
	peak viscosity mPa·s	time to reach peak viscosity h	average particle size μm	GM content %
KP	54,550	4.0	304	75.0
PA	109,100	7.0	315	98.1
S-P	34,020	0.5	105	97.0
S-gw	32,200	0.5	162	97.0

Data are representative of over five separate analyses.

Inhibitory Effects of Pulverized S-P on the Development of Dermatitis and Itching Behavior in NC/Nga Mice

The ameliorative effects of low-viscous S-P and S-gw on spontaneously occurring dermatitis in NC/Nga mice were examined to reveal new physiological functions while monitoring the clinical skin severity weekly. The weight gains of mice scarcely changed among the groups. However, increases in the weight of cecal contents were observed in NC/Nga mice fed the pulverized S-P diet: 0.42 ± 0.01 g (mean ± SE, n = 6) for control, 0.55 ± 0.08 g (n = 6) for KP, 0.50 ± 0.07 g (n = 6) for PA, 0.70 ± 0.03 g (n = 6) for S-P, and 0.46 ± 0.03 g (n = 3) for S-gw. As depicted in figure 1a, oral intake of low-viscous S-P markedly suppressed the increase in the clinical skin severity scores with time, although high-viscous KP feeding or PA feeding was ineffective in the development of these skin symptoms in NC/Nga mice. Scratching markedly increased in KP-fed and PA-fed mice from 7 weeks (fig. 1b). Similarly, the increased scratching behavior was profoundly inhibited in NC/Nga mice fed fine S-P. Surprisingly, oral intake of S-gw failed to prevent the development of dermatitis-like KP and PA feeding, while pulverized S-P induced a striking suppression (see S-gw group in fig. 1). In all cases, control mice showed behaviors similar to the KP-fed, PA-fed, or S-gw-fed mice.

Figure 2 indicates representative photographs of NC/Nga mice at 10 weeks. Any difference in the coat of NC/Nga mice fed S-P was not recognized, while the massive development of AD-like skin lesions (hemorrhage, loss of hair, edema, and excoriation) was observed in the mice fed the other GM diets as well as the control diet.

Thus, the low-viscous fine S-P was found to suppress the development of dermatitis in NC/Nga mice. Further-

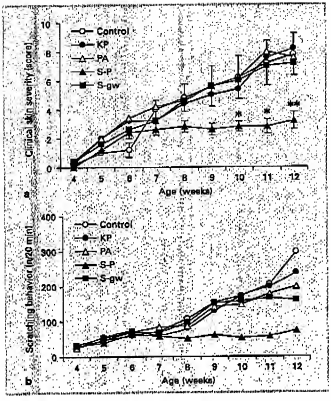

Fig. 1. Inhibitory effects of fine konjac GM on the increase in clinical skin scores and scratching behaviors in NC/Nga mice. **a** Clinical skin conditions in NC/Nga mice during the 8-week experiment. The skin severity score was evaluated by the procedures described in the Materials and Methods. Values are expressed as means ± SE of 5 mice per group. * $p < 0.05$, ** $p < 0.01$, vs. the control diet. **b** Scratching behaviors in NC/Nga mice during the 8-week experiment. The scratching behavior with the hind paws was evaluated by the procedures described in the Materials and Methods. Values are expressed as the total number of scratching incidents in 5 NC/Nga mice per 20 min.

more, the particle size rather than the viscosity was regarded as the main factor responsible for this physiological function.

Inhibitory Effects of Fine GM on Plasma IgE Elevation in NC/Nga Mice

A remarkable suppression of total plasma IgE elevation was observed in NC/Nga mice fed the S-P diet (fig. 3a). However, total plasma IgE levels in the other GM-fed mice as well as control mice gradually increased with aging. Total plasma IgG1 and IgG2a levels were examined at 12 weeks (fig. 3b, c, respectively). Total IgG1 levels in NC/Nga mice fed S-P were significantly lower than those fed the control diet. However, no statistically significant difference in total IgG2a levels were observed among the groups tested, although a similar tendency to a decrease was seen between IgG1 and IgG2a levels.

Cytokine Production of Splenic T Cells from S-P-Fed Mice

To gain more insight into the mechanism underlying eczema prevention by oral intake of pulverized S-P, we next tested the effect of oral intake of konjac GM powders on the Th1/Th2 cytokine expression by splenic T cells upon stimulation with immobilized anti-CD3 plus anti-CD28 antibodies. Both IL-4 and IFN-γ secretion by splenocytes tended to decrease in the S-P group (fig. 4), although the differences were not statistically significant compared to the control group. However, the suppressive effect of S-P was significant ($p < 0.05$) compared with the high-viscous KP or PA groups (fig. 5). The fact that pulverized S-P inhibits both Th1 and Th2 cytokine secretion implicates that the prevention of skin inflammation by fine S-P is not attributable to its effect on Th1/Th2 polarization.

Fig. 2. Representative photographs of NC/Nga mice at the age of 10 weeks. The left and right photographs represent the control group and pulverized GM group, respectively. AD-like skin symptoms were markedly suppressed by oral intake of pulverized GM.

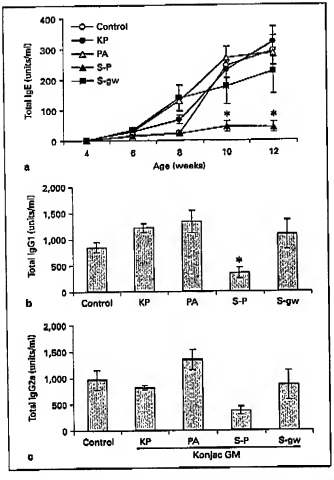

Fig. 3. Effects of konjac GM on total plasma IgE levels during the 8-week breeding, and on IgG1 and IgG2a levels in NC/Nga mice at 12 weeks of age. IgE (**a**), IgG1 (**b**), and IgG2a (**c**) were quantified by sandwich ELISA. Means ± SE of 5 mice for IgE (**a**), and of 3 mice for IgG1 (**b**) and IgG2a (**c**) per group. One unit is defined as 1 μg of mouse IgE, IgG1, or IgG2a standard. * $p < 0.05$ vs. the control diet.

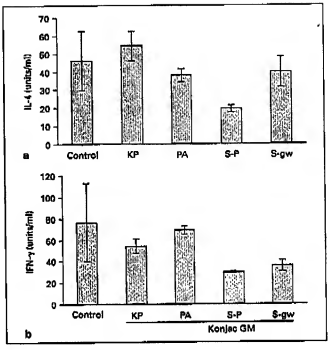

Fig. 4. Effects of konjac GM on the cytokine production of murine spleen T cells at 12 weeks of age. Spleen cells were stimulated with anti-CD3 plus anti-CD28 antibodies for 3 days. IL-4 (**a**) and IFN-γ (**b**) levels secreted in the culture supernatant were quantified by sandwich ELISA, using the reagents and method from PharMingen. One unit is defined as 1 pg of mouse IL-4 standard, or 1 ng of mouse IFN-γ standard. Means ± SE of 3 mice per group.

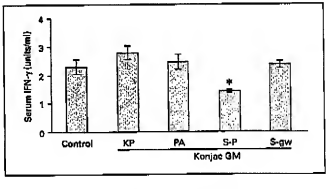

Fig. 5. Effects of konjac GM on the plasma IFN-γ levels in NC/Nga mice at 12 weeks of age. IFN-γ levels were quantified by sandwich ELISA. Means ± SE of 3 mice per group. One unit is defined as 1 ng of mouse IFN-γ standard. * $p < 0.05$ vs. the control diet.

Inhibitory Effects of Plasma IFN-γ Levels in NC/Nga Mice

Several lines of evidence have demonstrated the critical role of IFN-γ in the development of chronic AD [12–15, 17]. Combined with the decrease in IFN-γ secretion by splenic T cells from S-P-fed mice (fig. 4b), this evidence prompted us to examine the dietary effect of the different konjac GM powders on the plasma concentration of IFN-γ. As expected, in mice fed the S-P diet (12 weeks of age), plasma levels of IFN-γ were significantly lower compared to control mice, whereas this inhibitory effect was absent in mice fed the other konjac GM diets (fig. 5). Therefore, the systemic downregulation of IFN-γ could be one possible mechanism to prevent the development of AD-like skin inflammation.

Discussion

High-viscous konjac GM powders (KP and PA) have previously been described to play an important role in the prevention of diabetes and hypercholesterolemia [7]. The potency of various physiological activities depended upon the viscosity. Accordingly, the physiological function of low-viscous GM has not been the subject of research. By investigating the dietary effect of several low-viscous konjac GM powders on spontaneously occurring dermatitis in NC/Nga mice, we found that oral intake of low-viscous GM (S-P) with only small particle size restrained the progression of dermatitis, itching behavior, and plasma IgE elevation in NC/Nga mice (fig. 1a, b, 3a), while a suppressive effect of high-viscous GM (KP and PA) and low-viscous re-granulated fine GM (S-gw) on AD-like dermatitis was lacking. These results implied that the suppressive effect of konjac GM on the development of AD-like dermatitis should depend upon the particle size rather than the viscosity. These findings suggested that even the same substance could exhibit different physiological functions stemming from the differences in the shape and size of foodstuffs.

Dietary restriction of calories, proteins, vitamins, and minerals suppressed lymphocyte proliferation and Th2 cytokine and serum IgE production [18, 19] and delayed the onset and suppressed progression of AD-like dermatitis in NC/Nga mice [20]. However, no differences in body weight gain and food intake were observed among the NC/Nga mice. We therefore concluded that the suppressive effect was not caused by food restriction.

Colonic fermentation of finely ground wheat bran was reported to be superior to that of coarse wheat bran [21, 22]. Oral administration of high-viscous GM increased the weight of the cecum and cecal contents, reduced the pH value of the cecal contents, and altered IgA levels in the cecal contents and feces [23]. In the present study, the weight of cecal or colonic contents increased in NC/Nga mice fed konjac GM, especially in mice fed low-viscous fine S-P. Furthermore, the differences in physiological functions between fine S-P and S-gw on immunomodulatory activity may reflect the differences in the amounts of GM fermented or bacterial growth in the cecum and colon. Practically, the finer particle size of GM seems to be degraded more easily into oligosaccharides, because the increase in the specific surface area would promote bacterial adherence or enzymic access to the surface area of the GM molecule. Oral intake of konjac GM significantly enhanced fecal bifidobacteria in C3H/He male mice [24]. Kalliomäki et al. [25] have reported that atopic infants had more clostridia and tended to have fewer bifidobacteria in their feces than non-atopic infants, resulting in a reduced ratio of bifidobacteria to clostridia. In addition, the incidence of atopic eczema in infants given a probiotic was reduced by half as compared with those on a placebo when *Lactobacillus rhamnosus* GG strain was given prenatally to mothers and postnatally for 6 months to their infants at high risk of allergy [26]. Although it is unclear how gut microflora influences the immunomodulatory function, it may be suggested that such hydrolyzed GM and/or oligosaccharides may have an indirect effect on skin inflammation and systemic immune function by activating the intestinal immune system with the change in the colonic microflora.

In the future, we will have to elucidate the immunomodulatory activity of pulverized GM (S-P) on systemic and gut immune systems: the direct action of S-P, the direct function of hydrolyzed S-P, or the indirect effect of the rapidly growing microflora on the release of oligosaccharides.

The inhibitory effect of fine S-P on both Th1 and Th2 cytokine production (fig. 4) as well as on the respective immunoglobulin levels (fig. 3) implicates that S-P might serve as an immunosuppressant, which triggers T-cell unresponsiveness. However, our preliminary experiment shows that T-cell proliferation via T-cell receptor ligation normally occurs in splenic cells from S-P-fed mice (data not shown), suggesting that the downregulation of antibody/cytokine responses by S-P is not attributable to the impaired T-cell receptor signaling or T-cell unresponsiveness. We cannot elucidate the mechanism underlying the immunomodulatory action using NC/Nga mice, since no specific allergen has been identified in this mouse model.

Thus, a more detailed antigen-specific system is required to analyze the effect of the S-P diet on antigen-specific T-cell responses and dendritic cell function in the mucosa further.

Recent studies have demonstrated that IFN-γ production by Th1 cells plays an important role in the pathophysiology of AD. In a study by Grewe et al. [12], the increase in IFN-γ mRNA expression was significantly suppressed after successful therapy of AD patients regardless of a change in IL-4 mRNA expression. In NC/Nga mice, the Th2-mediated immune response might not be necessary for the development of AD-like skin disease [13]. Sumiyoshi et al. [14] showed that TGF-β_1 suppressed AD-like dermatitis in NC/Nga mice via downregulation of IFN-γ. Oral administration of royal jelly suppressed the development of AD-like skin lesions in picrylchloride-treated NC/Nga mice through a combination of downregulating TNP-specific IFN-γ production [15]. In a murine model of allergen-triggered AD, IFN-γ-deficient mice show decreasd chronic skin hypertrophy [17]. These findings suggest that IFN-γ production may be associated with the development of dermatitis in NC/Nga mice. In fact, fine S-P was able to downregulate IFN-γ production in the systemic immune system (fig. 4, 5). We therefore suggest that the inhibitory effect of pulverized GM on the development of dermatitis in NC/Nga mice might be associated with the downregulation of IFN-γ.

Transgenic mice expressing IL-18 in keratinocytes that had completely impaired Th2 responses elicited AD-like symptoms to the same extent as control mice [27]. In addition, it has been reported that serum IL-18 levels in patients with AD and NC/Nga mice were overexpressed [28]. Since IL-18 in the presence of IL-12 synergistically induces IFN-γ production from T and B cells without involving their antigen receptors [29], oral intake of pulverized GM might influence IL-18 production in the innate immune response.

In conclusion, the present study demonstrated that oral intake of pulverized GM with low viscosity inhibited the development of dermatitis and plasma IgE elevation in NC/Nga mice through the downregulation of IFN-γ production. The precise mechanisms responsible for the inhibitory effect of fine GM on the development of dermatitis are not clear so far. Further studies are necessary to reveal the role of these substances in immunomodulation.

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